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Urinary autoantibodies against intrinsic factor in pernicious anaemia patients

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Summary. In radioimmunoassay seven concentrated urines of pernicious anaemia (PA) patients were positive for intrinsic factor (IF). Four were studied by gel filtration. Two contained both binding and blocking antibodies against IF, one had only blocking antibodies and one lacked both types of antibodies. The antibodies were mainly of the IgG-type. No such

antibodies were found in the urine of a healthy person. None of the urines studied contained enough protein to be classified as proteinuric. Not until the interferences of the autoantibodies in the IF assay can be eliminated is the assay of value in the diagnosis of PA.

Pernicious anaemia (PA) is an autoimmune disease. The patients usually have circulating antibodies against both parietal cells and intrinsic factor (IF) (Chanarin, 1979). There are two types of IF antibodies: the blocking antibodies inhibit the binding of cobalamin (Cbl) to IF and the binding antibodies bind the Cbl-IF complex. The blocking antibodies mainly bind free IF but some also liberate Cbl from the Cbl-IF complex (Schade *et al.*, 1967).

A radioimmunoassay (RIA) detected small amounts of urinary IF (uro-IF) in healthy persons but not in pernicious anaemia patients (Gräsbeck *et al.*, 1982). Immunoreactive IF was not found in the sera of healthy persons but some PA sera were spuriously positive due to autoantibodies against IF (Gräsbeck *et al.*, 1983). Subsequently it was noted that some concentrated PA urines were positive for the same reason. Because specific urinary autoantibodies have previously been detected only in a few autoimmune diseases, it is interesting to study the urinary antibodies against IF.

MATERIALS AND METHODS

Reagents. The substances and their sources were: cyanocobalamin (Cbl, 738 $\mu\text{mol/l}$) (Organon, Netherlands), ^{57}Co -CN-Cbl (220 $\mu\text{Ci}/\mu\text{g}$) (Amersham, England), adenine cyanocobamide (pseudovitamin B₁₂) (MSD, N.J.), aprotinin (Sigma, Mo., or Medica, Finland), goat anti-rabbit IgG serum (Cambridge Medical Diagnosis, Mass.), hog anti-human IgG and anti-human IgA (Orion, Finland). Our anti-human IF

has been described, present serum was taken from the rabbit 14 d later (Gräsbeck *et al.*, 1982; Wahlstedt *et al.*, 1983).

RIA. The procedure was modified from that of Wahlstedt *et al.* (1983). The assay buffer was 0.1 M phosphate, pH 7.4, containing 0.15 M NaCl, 0.5% bovine serum albumin (BSA) and 0.01% NaN₃. 100 μl sample or standard (diluted human gastric juice), 20 μl non-radioactive CN-Cbl (3.7 pmol), 100 μl ^{57}Co -Cbl-IF (6.8 fmol IF-bound Cbl in gastric juice) and 100 μl of the mixture of ordinary rabbit serum (1:400) and rabbit anti-human IF-serum (1:250), or buffer for the individual blank tubes, were incubated at 4°C in the dark for 17–24 h. 100 μl of the second antibody (goat anti-rabbit IgG serum, 1:25) and 100 μl PEG (2 g/10 ml H₂O) were added. The incubation was continued at 20°C in the dark for 2 h. The mixture was centrifuged for 10 min at 1500 g and the precipitate counted. The radioactivity bound to the antibody was calculated as per cent of the total and expressed as moles Cbl bound to IF (IF binds Cbl in a 1:1 molar ratio). The same protocol was used to determine the titre of binding antibodies in a patient serum and concentrated urine.

Urines. Overnight urines were collected. NaN₃ and aprotinin (0.02% and 5000 kIU/l, respectively) were added and the urines were concentrated by ultrafiltration and dialysed against 0.01 M phosphate buffer, pH 7.4, containing 0.012% NaN₃ (Gräsbeck & Kouvonen, 1983). Protein was measured (Lowry *et al.*, 1951) using human serum albumin as standard and uro-IF was measured by RIA. Some urines collected after pentagastrin stimulation were used: After overnight fasting 6 μg pentagastrin/kg body weight (Peptavlon, 0.25 mg/ml, ICI, U.K.) was injected s.c. and urine was collected for 2 h. The mean concentration factor was 213 (extremes 53–390, $n=19$).

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Table I. Gel filtrations of concentrated control urine. Unconc: urine concentrate. $^{57}\text{Co-Cbl}$: 0.35 pmol if not stated otherwise. GJ: 5 μl of human gastric juice if not stated otherwise. AntilgG/antilgA: 10 μl hog anti-human IgG or IgA (no free Cbl-binding capacity). PEG: 50 μl of 20% polyethylene glycol. s: supernatant of the urine concentrate after the precipitation with antilgG/antilgA + PEG. AntilgG/antilgA + PEG precipitations were performed at 4°C using 100 or 150 μl of 0.1 M phosphate-0.15 M NaCl-0.01% NaN_3 buffer, pH 7.4. Other incubations were performed at 20°C in 0.1 M phosphate-1.0 M NaCl-0.01 M NaN_3 buffer, pH 7.4, in a total volume of 500 μl (1 ml after the precipitations). (HC=haptocorrin, TC=transcobalamin).

Incubation	Cbl-binding capacity (ng/incubation)		
	V_0	HC + IF + TC	Total
Urine concentrate: 100 μl			
GJ + $^{57}\text{Co-Cbl}$	<0.001	0.126	0.126
Unconc + $^{57}\text{Co-Cbl}$	<0.001	0.019 + 0.030	0.049
Binding			
(GJ + $^{57}\text{Co-Cbl}$) + Unconc	0.002	0.271	0.273
[(GJ + $^{57}\text{Co-Cbl}$) + Unconc] + antilgG	0.003	0.280	0.283
(Unconc + antilgG + PEG)s + (GJ + $^{57}\text{Co-Cbl}$)	0.001	0.213	0.214
Blocking			
(GJ + Unconc) + $^{57}\text{Co-Cbl}$	0.001	0.235	0.236
[(Unconc + antilgG + PEG)s + GJ] + $^{57}\text{Co-Cbl}$	0.001	0.237	0.238

Table IIA. Gel filtrations of concentrated (220-fold) urine of a pernicious anaemia patient (I.G.). (Explanations in Table I)

Incubations	Cbl-binding capacity (ng/incubation)		
	V_0 (% of total)	HC + IF + TC	Total (% lost from original)
Urine concentrate: 100 μl			
GJ + $^{57}\text{Co-Cbl}$	<0.001	0.136	0.136
Unconc + $^{57}\text{Co-Cbl}$	<0.001	<0.001	0.001
Binding			
(GJ + $^{57}\text{Co-Cbl}$) + Unconc	0.025 (18%)	0.113	0.138
(GJ + $^{57}\text{Co-Cbl}$) + Unconc + antilgG	0.041 (23%)	0.134	0.175
Urine concentrate: 50 μl			
5 μl GJ + $^{57}\text{Co-Cbl}$	<0.001	0.109	0.109
20 μl GJ + 0.53 pmol $^{57}\text{Co-Cbl}$	<0.001	0.357	0.357
Unconc + $^{57}\text{Co-Cbl}$	0	0	0
Binding			
(GJ + $^{57}\text{Co-Cbl}$) + Unconc	0.012 (13%)	0.076	0.088
[(GJ + $^{57}\text{Co-Cbl}$) + Unconc] + antilgA	0.015 (16%)	0.077	0.092
Blocking			
(GJ + Unconc) + $^{57}\text{Co-Cbl}$	<0.001	<0.007	0.007 (94%)
(20 μl GJ + Unconc) + 0.53 pmol $^{57}\text{Co-Cbl}$	0.014 (5.8%)	0.226	0.240 (33%)

Gel filtrations. Gel filtration was performed at 20°C in a Sephadex G-200 column (15 × 800 mm) equilibrated with 1.0 M NaCl, 0.1 M phosphate and 0.01% NaN_3 , pH 7.4 (Wahlstedt & Gräsbeck, 1985). One patient urine was examined with a similar Sephacryl S-200 column and a patient serum with Sephacryl S-300 HR column with 0.05% Triton X-100 in the buffer. See Tables I, IIA and IIB for incubation protocols.

Cbl-binding capacity. Concentrated urine, human gastric juice and serum were incubated with $^{57}\text{Co-Cbl}$ for 30–60 min to saturate the Cbl-binding proteins and passed through the Sephadex column. In some experiments pseudo- B_{12} (60-fold excess) was added to the urine concentrate to saturate the non-IF-related Cbl-binding. The Cbl-binding capacity of the binders was calculated from the radioactivity of the peaks emerging before the total volume of the column had been eluted.

Table IIB. Gel filtrations of concentrated (110-fold) urine of a pernicious anaemia patient (I.G.). (Explanations in Table I)

Incubations	Cbl-binding capacity (ng/incubation)		
	V_0 (% of total)	HC + IF + TC	Total (% lost from original)
Urine concentrate: 50 μ l			
GJ + $^{57}\text{Co-Cbl}$	<0.001	0.074	0.074
Unconc + $^{57}\text{Co-Cbl}$	0	0	0
Binding			
(GJ + $^{57}\text{Co-Cbl}$) + Unconc	0.020 (18%)	0.090	0.110
(Unconc + 5 μ l antiIgG + PEG)s + (GJ + $^{57}\text{Co-Cbl}$)	0.004 (3.4%)	0.112	0.116
(Unconc + 5 μ l antiIgA + PEG)s + (GJ + $^{57}\text{Co-Cbl}$)	0.021 (14%)	0.125	0.146
Unconc + PEG)s + (GJ + $^{57}\text{Co-Cbl}$)	0.015 (15%)	0.087	0.102
Blocking			
(GJ + Unconc) + $^{57}\text{Co-Cbl}$	0.003	0.017	0.020 (73%)
[(Unconc + 5 μ l antiIgG + PEG)s + GJ] + $^{57}\text{Co-Cbl}$	0.001	0.069	0.070 (5.4%)
[(40 μ l Unconc + 5 μ l antiIgA + PEG)s + GJ] + $^{57}\text{Co-Cbl}$	0.003	0.025	0.028 (62%)
[(Unconc + PEG)s + GJ] + $^{57}\text{Co-Cbl}$	0.001	0.010	0.011 (85%)

Demonstration of antibodies. Urine concentrate was incubated with gastric juice for 2 h, $^{57}\text{Co-Cbl}$ was added, and after 30 min the sample was filtered through the Sephadex column. Diminution of the IF-bound radioactivity indicates the presence of blocking antibodies. Gastric juice was saturated with $^{57}\text{Co-Cbl}$, urine concentrate was added and after 2 h the sample was filtered or pig anti-human IgG or IgA was added for further 2 h before gel filtration. Movement of the IF-bound radioactivity to a position close to the totally excluded volume (V_0) indicates the presence of binding antibodies. Binding of second antibody sharpens the peak near the V_0 and moves it closer to V_0 .

Precipitation of immunoglobulins before demonstration of antibodies. Anti-human IgG or IgA (or buffer for controls), 20% polyethylene glycol and 0.1 mol/l phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.01% NaN_3 , were added to the urine concentrate. The mixture was incubated at 4°C for 1.5 h and then centrifuged at 1000 g in a Beckman Microfuge B for 1 min. The supernatant was used in the incubations instead of concentrated urine. If $^{57}\text{Co-Cbl-IF}$ is not transferred to V_0 or the Cbl-binding capacity of IF is not blocked, this indicates that the mixture of anti-Ig and PEG (final concentration 3.8%, as in the uro-IF RIA) has precipitated the anti-IF-antibodies (Creighton *et al.*, 1973).

Serum autoantibodies. Blocking antibodies were determined by the haemoglobin-coated charcoal method (Samloff *et al.*, 1968) in the Medix Laboratories, Espoo, Finland.

Patients. Uro-IF was assessed on six healthy persons (controls) and seven PA patients, aged 39–80 years, outpatients of Maria Hospital, Helsinki, duration of diagnosed PA 0–17 years. The criteria for PA were moderate to severe gastric atrophy, macrocytic anaemia, low Cbl, pepsinogen I

and high gastrin in serum, response to treatment with Cbl, and low Schilling test, increasing with IF. Informed consent and approval by the ethical committee were obtained. Gel filtrations were performed with concentrates of one control and four PA patients.

RESULTS

Unspecific binding of labelled IF in RIA

Non-specific binding was $1.9 \pm 0.4\%$ (standard deviation, SD; $n = 14$) for a standard series (containing 0 pmol IF and no anti-IF-serum) and 2.5 ± 1.1 (SD, $n = 21$) for urine samples from healthy controls. The mean uro-IF concentration of six healthy persons determined without individual blanks was 2.48 pmol/l original urine. Several concentrates of PA patient urine had an increased unspecific binding; i.e. 2.7–12.3% (mean 5.1%, $n = 7$). The mean uro-IF concentration was 3.24 pmol/l when calculated from individual blanks and 1.99 pmol/l from standard series blanks, the difference being 0.26–4.13 pmol/l.

Gel filtrations

The studies were mainly performed using concentrated urine (Unconc). The concentration factor is provided in parentheses, e.g. (128 \times) = 128-fold concentration.

Unconc of control

In the binding experiments there was no transfer of radioactivity from IF to the V_0 (Table I, Fig 1). In the blocking studies there was no diminution in the Cbl-binding capacity of IF. Precipitation with anti-human IgG and PEG did not clearly

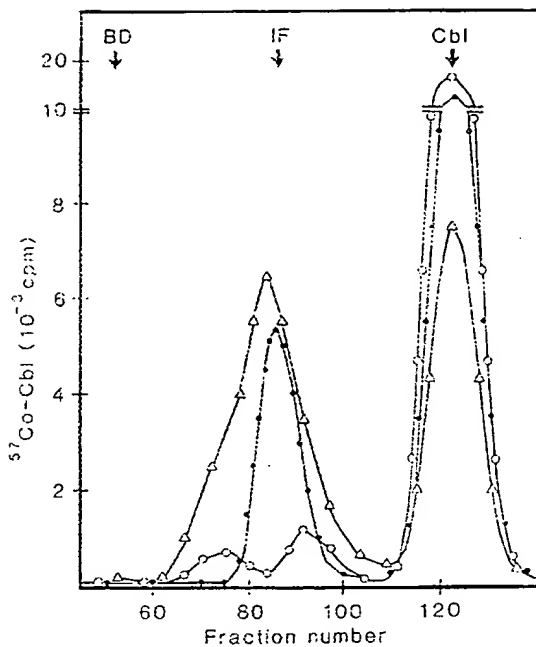


Fig 1. Gel filtration of human gastric juice IF with ^{57}Co -Cbl before (●) and after (Δ) incubation with a concentrated urine of a healthy person. Filtration of this urine concentrate with ^{57}Co -Cbl (○). (From Table I.) The arrows indicate V_0 (Blue Dextran, BD), human gastric juice intrinsic factor (IF) and free cobalamin (Cbl).

change the Cbl-binding capacities. Indicating that antibodies against IF were not present in control urine.

In all incubations of gastric juice with Unconc the total ^{57}Co -Cbl-binding capacity was about 140% of the expected capacity calculated from separate analyses of Unconc (haptocorrin and transcobalamin peaks) and gastric juice (see Fig 1). The ^{57}Co -Cbl-IF peak also became somewhat wider and the apparent molecular weight of IF slightly increased (about 14 000 daltons).

Unconc of a patient (I.G.)

Patient I.G. had had PA for 4 years and her serum contained blocking IF-antibodies. In the binding experiment the Unconc (220 ×, 100 μl) caused the transfer of 18% of the total protein-bound radioactivity to the V_0 . Addition of anti-human IgG slightly increased this transfer (23%) and sharpened the V_0 peak (Table IIA, Fig 2). Anti-human IgA caused a smaller transfer in a 50 μl sample of the Unconc (Table IIA). The elution volume of ^{57}Co -Cbl-IF was slightly decreased (Fig 2).

Precipitation of another Unconc (110 ×, after pentagastrin stimulation) with anti-human IgG and PEG but not with anti-human IgA and PEG or PEG alone clearly diminished the transfer of the bound radioactivity to the V_0 . The results (Table IIB) indicate the presence of binding IgG-type antibodies against IF.

In the blocking experiments the former Unconc (50 μl) decreased the bound radioactivity of 5 μl and 20 μl of gastric juice by 94% and 33%, respectively. When using 20 μl of gastric juice, 5.8% of the bound radioactivity was found in

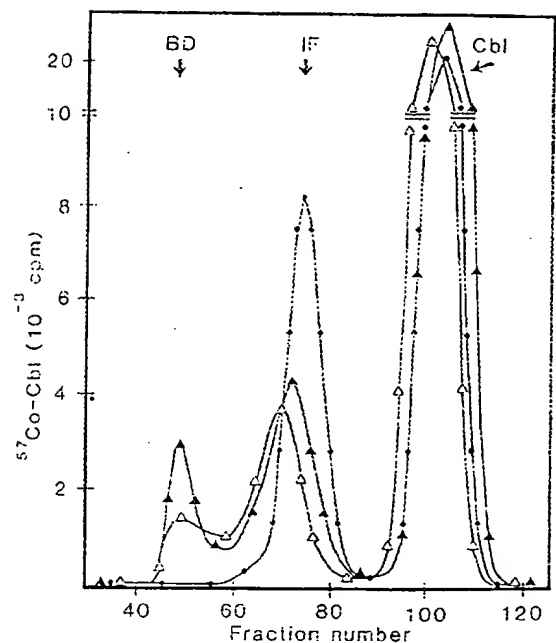


Fig 2. Gel filtration of gastric juice IF with urine concentrate of a pernicious anaemia patient (I.G.). The urine contains type II or binding antibodies against IF. (From Table IIA, 100 μl urine concentrate.) ^{57}Co -Cbl-saturated gastric juice (●). Incubation of ^{57}Co -Cbl saturated gastric juice with the urine concentrate (Δ). Note transfer of ^{57}Co -Cbl-IF to the V_0 . Continuing the incubation with anti-human IgG increases the V_0 -peak (Δ).

the V_0 . The results (Table IIA) indicate the presence of binding antibodies.

The other Unconc decreased the bound radioactivity by 73% (Table IIB, Fig 3). After precipitation with anti-human IgG and PEG, the decrease was only 5.4%, but with anti-human IgA and PEG or PEG alone 62% and 85%, respectively. The blocking antibodies evidently belong to the IgG-class. A third Unconc (265 ×, 24 h urine) was titrated in the RIA. It bound the ^{57}Co -Cbl-IF maximally in the dilution 1:16 (0.082 fmol/μl concentrate).

Serum of patient I.G.

5 μl serum (0.005 ng Cbl-binding capacity) transferred 69% of the protein bound ^{57}Co -Cbl to the V_0 before and 25% after precipitation with anti-IgG + PEG; in both cases the total ^{57}Co -Cbl-binding capacity increased. The same amount of serum blocked 85% of 5 μl gastric juice before and 67% after the precipitation. When titrated in the RIA, the serum bound the tracer maximally in the final dilutions 1:160 and 1:320; the dilution 1:800 bound 0.009 pmol IF/μl serum. When titrated for blocking antibodies with the charcoal method 1 μl serum blocked 0.382 pmol IF. Evidently, this serum contained binding and blocking antibodies.

Patient E.M.L.

Patient E.M.L. had newly diagnosed PA and her serum contained blocking antibodies. The Unconc (312 ×) had high Cbl-binding capacity: the HC and TC peaks bound together

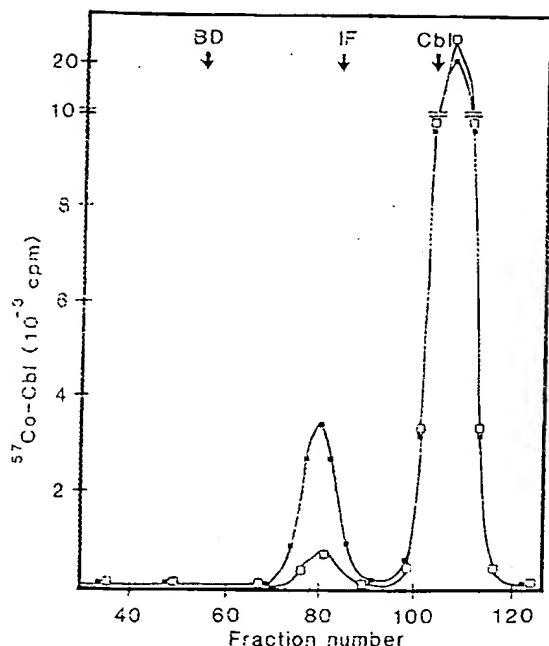


Fig 3. Gel filtration of gastric juice with a urine concentrate of a pernicious anaemia patient (I.G.). (From Table IIB.) The urine contains type I or blocking antibodies. (a) The gastric juice was incubated with the patient's concentrated urine before saturation with $^{57}\text{Co-Cbl}$ (□). The $^{57}\text{Co-Cbl}$ -binding capacity of IF is low. (b) The urine concentrate was incubated with anti-human IgG and PEG and centrifuged. The supernatant was used instead of the original concentrate in the experiment described under (a) (■). The $^{57}\text{Co-Cbl}$ -binding capacity of IF is not decreased. If the anti-human IgG is omitted, the supernatant blocks the $^{57}\text{Co-Cbl}$ -binding capacity of IF.

10.0 pmol/ml. 125 μl of the concentrate, treated with pseudo- B_{12} , failed to transfer radioactivity from $^{57}\text{Co-Cbl-IF}$ to the V_0 and to decrease the elution volume of $^{57}\text{Co-Cbl-IF}$. Two other urines (230 \times , overnight urine and one 96 \times , after pentagastrin) did not block the Cbl-binding of IF. Thus binding and blocking antibodies were not detected.

Patient T.L.

Patient T.L. had had PA for 4 years and blocking antibodies in serum. Unconc (200 \times) had a Cbl-binding capacity (0.191 pmol/ml) eluting as TC. In the binding experiment, the total Cbl-binding capacity did not increase significantly (4.4%), only 2.2% of it moved to the V_0 . After incubation with anti-human IgG, the V_0 peak became somewhat sharper. 50 μl Unconc blocked 97% of the $^{57}\text{Co-Cbl}$ -binding capacity of 5 μl gastric juice and 33% of 20 μl gastric juice. Treatment with anti-IgG and PEG did not change the binding activity but inhibited the blocking activity completely. After treatment with anti-IgA and PEG the Unconc still blocked 54% of the Cbl-binding capacity of 5 μl of gastric juice. The results indicate the presence of blocking antibodies of the IgG-type, possibly also of IgA. The presence of binding antibodies is uncertain.

Patient E.C.

Patient E.C. had had PA for 17 years and blocking antibodies in serum. The Unconc (152 \times) had a Cbl-binding capacity of

only 0.085 pmol/ml, located in the TC peak. In the binding experiment the calculated total binding capacity increased by 15%, there was a transfer of 19% of this activity to the V_0 , the Cbl-IF peak became clearly wider and its elution volume decreased somewhat. Another Unconc (53 \times , after pentagastrin), showed a 10% increase of the total Cbl-binding capacity, a transfer of 6% of the radioactivity to the V_0 and a decrease in the elution volume of IF. Evidently the urines contain binding antibodies.

In the blocking experiment, 50 μl of the latter Unconc decreased the $^{57}\text{Co-Cbl}$ -binding capacity of 5 μl of gastric juice by 90% and that of 20 μl by 55%. In both cases about 3.5% of the ^{57}Co was found in the V_0 . This Unconc did not bind Cbl at all. The V_0 fractions from the blocking experiment performed with 5 μl of gastric juice, after dialysis (pH 5) and concentration by ultrafiltration, exhibited no Cbl-binding capacity (pH 7.4) but decreased the Cbl-binding capacity of 5 μl of gastric juice by 48%. The concentrate from the V_0 most likely did not contain free endogenous Cbl (ultrafiltration would have removed it). Apparently the urine contained blocking antibodies and endogenous Cbl was not the blocking agent.

DISCUSSION

In the preliminary study on uro-IF, where the urines were concentrated about 50-fold, only few PA urines were false positive (Gräsbeck *et al.* 1982). Now several patient urines, concentrated about 200-fold, exhibited high unspecific binding and appeared to be positive for IF by RIA. The human anti-IF antibodies evidently bound the tracer in the same way as the rabbit antibodies and precipitation occurred with the second antibody, which crossreacts with human IgG. In gel filtration for antibodies against IF, these concentrates behaved in the same way as sera containing anti-IF antibodies (Garrido-Pinson *et al.* 1966; Marcoullis *et al.* 1979).

In conclusion, the concentrated urines of seven PA patients were positive for IF in the RIA. Four of these were studied by gel filtration, two contained both binding and blocking antibodies, one had only blocking antibodies and one lacked both types of antibodies. The urine of the healthy control did not contain antibodies. This is the same pattern of antibodies that patient sera are known to exhibit (Samloff *et al.* 1968; Conn, 1986).

The antibodies were mainly of the IgG type. Of the circulating antibodies, those with blocking activity have been shown to be IgG and those with binding activity IgG and IgM (Marcoullis *et al.* 1979; Conn, 1986). In gastric juice the antibodies are mainly IgG, but secretory IgA has also been demonstrated (Goldberg & Bluestone, 1970). The salivary antibody of one patient was IgA (Carmel & Herbert, 1976). The blocking antibody activity found here was not due to free endogenous Cbl. Large amounts of endogenous Cbl cause a falsely positive IF-antibody reaction because the Cbl-binding capacity of IF is blocked (Stenman, 1976).

The urine and serum of one patient (I.G.) were compared. Both contained binding and blocking antibodies, but the titre was much lower in the urine. The urine of another patient (E.M.L.) whose serum contained blocking antibodies, gave a positive reaction in RIA but antibodies were not detected by

gel filtration. As the PA of this patient was newly diagnosed, the urine may still have contained some IF or an undetectably small amount of antibodies. Carmel (1982) reported a patient in whose serum blocking antibody activity gradually appeared coinciding with the conversion of his Schilling test to pathological.

Few reports exist on the excretion of specific autoantibodies. Antinuclear antibodies (ANA) were found in concentrated proteinuric and non-proteinuric urines of patients with systemic lupus erythematosus (SLE), with mixed type connective tissue disease (Hanson & Tan, 1965) and progressive systemic sclerosis (PSS) (Meryhew *et al.* 1983) and in urine and pleural fluid of one SLE patient with profound proteinuria (Persellin & Takeuchi, 1980). Urinary thyroid stimulating immunoglobulin was found in one patient with Graves' disease and nephrotic syndrome (Kajita *et al.* 1980).

The urinary autoantibodies do not seem to be associated with kidney disease. Even under physiological conditions, urine contains a wide variety of plasma proteins, mainly albumin but also immunoglobulins, especially IgG and IgA (Berggård, 1970). Autoantibodies are filtered in the glomeruli as are other immunoglobulins, even though the glomeruli are intact and there is no pathological proteinuria. The renal clearance of IgG is only slightly lower than that of albumin. The source of urinary IgA is dual: filtered plasma and tubular secretion (Burdon, 1971). However, increasing excretion of specific antibodies could be a sign of altered glomerular permeability (Meryhew *et al.* 1983). Proteinuria is often found in autoimmune disorders, caused by immunocomplex deposition in the glomeruli or other mechanisms (Waller *et al.* 1989), but proteinuria is not a typical finding in PA (Chanarin, 1979). None of the urines studied here indicated proteinuria, defined by a total protein excretion above 150 mg/24 h (Waller *et al.* 1989).

In most gel filtrations of concentrated urine with ^{57}Co -Cbl-IF, the total protein-bound Cbl-activity increased and the IF peak broadened and was eluted earlier. The cause for this is unclear. Dissociation of endogenous Cbl from HC could explain the increase of the total Cbl-binding capacity in the elution volume of HC. Unpublished results suggest that uro-IF is strongly aggregated in concentrated urine. Added ^{57}Co -Cbl-IF could perhaps dissolve these aggregates and increase the Cbl-binding capacity of uro-IF; at least in the control urine. Only part of the urinary IF is able to bind Cbl (Wahlstedt & Gräsbeck, 1985).

As in the uro-IF RIA, the autoantibodies have been reported also to cause interferences in the RIA of free thyroxine (Konishi *et al.* 1982). Before the uro-IF test can be used for the diagnosis of PA these interferences must be eliminated.

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